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The Soy Isoflavone Genistein Enhances IFN-γ-Induced PD-L1 Expression in B16F1 Melanoma Cells *in Vitro*

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PD-L1 molecules on a tumor have attracted attention because PD-L1 on a tumor plays an important role in escape for host immune responses. It has been shown that genistein attenuates immune checkpoint therapy for B16F1 melanoma in mice. We examined the effect of genistein on expression of PD-L1 in B16F1 melanoma cells and found that genistein increases the expression of IFN-γ-induced PD-L1 molecules at both protein and mRNA levels. Genistein increased the mRNA expression of STAT1 and STAT3 in IFN-γ-treated B16F1 cells. We compared the effects of ten types of flavonoids on PD-L1 expression and found that genistein is a strong inducer of PD-L1 expression among the flavonoids.

Key words soy isoflavone, genistein, flavonoids, PD-L1, B16F1 melanoma

INTRODUCTION

Programed cell death 1 (PD-1), which belongs to the B7/CD28 superfamily, is a receptor expressed on the surface of T, B and NK cells that regulates cell activation. Its ligand, programmed cell death ligand 1 (PD-L1), is expressed in some tumor cells.[1] Cancer immune checkpoint therapy is a novel clinical strategy for cancer. It has been shown that cancer immune checkpoint therapy is useful for many types of cancer including metastatic melanoma, non-small cell lung cancer, renal cell carcinoma, and bladder or urothelial cancer.[2] Immune checkpoint blockade is based on inhibition of binding between PD-1 and PD-L1. PD-L1 molecules expressed on the tumor bind to PD-1 on T cells, resulting in suppression of T cell function.[3] Blocking of the PD-1/PD-L1 pathway with monoclonal (m) antibodies is a promising therapeutic approach. A meta-analysis of cancer immune checkpoint therapy has shown that a high expression level of PD-L-1 is a negative prognosis marker of various cancers.[4-6] The regulatory mechanism of PD-L1 on a tumor is therefore a crucial point for consideration of preventive and therapeutic approaches for cancer.

Soy is a rich source of multiple classes of bioactive components including the isoflavones genistein, daidzein and equol. Soy intake has been shown to prevent hormone-related cancers such as breast cancer and prostate cancer in humans.[7,8] Soy isoflavones are candidates for preventive components. It has been shown that the soy isoflavone genistein inhibits *in vitro* proliferation of tumor cells.[9] Treatment with genistein in mice that had been inoculated with B16F1 melanoma enhanced NK and cytotoxic cell activity that resulted in the suppression of tumor growth.[10]

The combination of immune checkpoint inhibitors with natural products may provide a novel strategy for treatment of tumors. It has been shown that the soy isoflavone equol activates $CD8^+$ T cells and improves the effect of immune checkpoint therapy.[11] In another study, it was shown that genistein attenuates the effects of immune checkpoint therapy.[12] In this study, we examined the effect of genistein on the expression of PD-L1 in B16F1 melanoma cells and we compared the regulatory effect of genistein with the regulatory effects of other flavonoids.

MATERIALS AND METHODS

Reagents Nobiletin, natudaidain and heptametoxyflavonoid were provided by Ushio-Chemix Co. (Shizuoka, Japan). Sudachitin was provided by Ikeda-Yakusou Co. (Tokushima, Japan). Demethoxysudachitin was provided by Hitach Chmical Co. (Hitachi, Japan). Quercetin, hesperidin, genistein, daidzein, and equol were purchased from Funakoshi Co. (Tokyo, Japan).

Flow Cytometric Analysis B16F1 cells were treated with 25 U/ml murine IFN-γ with or without 25 µM of each flavonoid in a 48-well plate for 24 h at 37°C under 5% CO_2. The B16F1 cells were stained with PE-conjugated anti-mouse PD-L1 m antibody (Ab) (clone: 10F.9G2) (eBioscience, CA, USA) and FITC-conjugated anti-mouse I-A mAb (clone: M5/114.15.2) (eBioscience). Flow cytometric analysis was performed on Guava easyCyte using Guava Incyte software (Merck Millipore, Darmstadt, Germany).

Reverse Transcription-Quantitative PCR (RT-qPCR) Total RNA was isolated from B16F1 cells using an RNAiso (Takara Bio, Siga, Japan). Five thousand ng of the extracted total RNA was transcribed by using a PrimeScript RT Master Mix kit (Takara Bio.). Real-time PCR was performed by using specific primers and SYBR green dye (Applied Biosystems, CA, USA) in ABI StepOnePlus™ (Applied

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Biosystems) according to the manufacturer's instructions. The primers used in this study were as follows:

Murine PD-L1 forward primer 5'-TGCTGCATAATCAGCTACGG-3';

Murine PD-L1 reverse primer 5'-TTCATGCTCAGAAGTGGCTG-3';

Murine STAT 1 forward primer 5'-GCCTCTCATTGTCACCGAAGAAC-3';

Murine STAT1 reverse primer 5'-TGGCTGACGTTGGAGATCACCA-3';

Murine STAT3 forward primer 5'-AGGAGTCTAACAACGGCAGCCT-3';

Murine STAT3 reverse primer 5'-GTGGTACACCTCAGTCTCGAAG-3';

Murine β-actin forward primer 5'-CTGACCCTGAAGTACCCCATTGAACA-3';

Murine β-actin reverse primer 5'-CTGGGGTGTTGAAGGTCTCAAACATG-3'.

Western Blot Analysis B16F1 cells was lysed with lysate buffer (150 mM Tris-HCl (ph 7.4), 0.5 mM EDTA, 10 mM NaF, 1% NP-40, 1 mM phenylmethylsulphonyl fluoride). Proteins were loaded on SDS-PAGE and then transferred onto polyvinylidene fluoride membranes. The membranes blocked with 5% nonfat milk were probed with primary Abs at 4°C overnight and then incubated with a horseradish peroxidase-conjugated secondary Ab. Rabbit anti-PD-L1 (cat. #2177812) and mouse anti-β actin (cat. #66009) were purchased from Bioss (MA, USA) and Proteintech (IL, USA), respectively.

PD-L1 Gene Promoter Reporter Assay The murine PD-L1 promoter region (- 2 kb) was amplified by PCR with specific primers (5'-GGGGTACCCCCTGGCTTAATCCTCACCTCTC-3' (sense) and 5'-GGGGTACCCCCCTGGTCTTATCTCGAGTTC-3' (antisense)) that contain unique *Kpn I* and *Bgl II* restriction enzyme sites, respectively. These fragments were cloned into the PGV-P2 vector (TOYO IN GROUP, Tokyo, Japan). B16F1 cells were transfected with the PGV-P2 vector containing the PD-L1 promoter region with the FuGENE HD transfection reagents (Roche, Mannhein, Germany) and treated with 25 U/ml IFN-γ and 25 μM of genistein for 24 h. Luciferase activity was determined by using a luminometer. The activitiy was calculated by adjustment of β-galactosidase activity as an internal control.

Statistical Analysis Data shown are representative of at least two or three experiments. Data were analyzed using Student's t-test for comparison to the IFN-γ-treated group. Data are expressed as means ± SD. Differences were considered significant at $p < 0.05$.

RESULTS

Genistein Enhances IFN-γ-Inducing PD-L1 Expression First, we examined the effect of genistein on the expression of PD-L1 in B16F1 melanoma cells by flow cytometric analysis (Fig. 1A). Treatment of B16F1 cells with IFN-γ induced the expression of PD-L1 molecules and their expression was further enhanced by treatment with genistein as evaluated by mean fluorescence intensity (MFI) (Fig. 1 B). We also examined the expression of MHC class II molecules, which is known to be induced by IFN-γ treatment. Genistein tended to decrease the expression of MHC class II molecules on IFN-γ treated B16F1 cells as evaluated by the percentages of positive cells and MFI (Fig. 1 A and C). We further examined the expression of PD-L1 by Western blot analysis and quantitative PCR analysis. The results showed that treatment with genistein further induces protein expression PD-L1 at a dose of 25 μM and mRNA expression of PD-L1 at doses of 12.5 and 25 μM in IFN-γ-treated B16F1 cells (Fig. 2A and B). We evaluated the effect of genistein on PD-L1 expression by a reporter assay. IFN-γ treatment induced PD-L1 promoter-driven luciferase activity in B16F1 cells. The activity was further increased by treatment with genistein (Fig. 2C).

Genistein Enhances STAT1 and STAT3 mRNA Expression Molecules of the transcription factor STAT1 and STAT3 have been shown to play a role in IFN-γ-induced PD-L1 on tumors.[13] We examined the expression of STAT1 and STAT3 molecules by quantitative PCR analysis. Treatment of IFN-γ induced both STAT1 and STAT3 mRNA expression, and treatment with IFN-γ and 12.5 μM of genistein further increased their expression levels (Fig. 3).

Genistein Is a Strong Inducer of IFN-γ-Induced PD-L1 Expression among the Flavonoids Tested Genistein a soy isoflavone. Flavonoids are classified as flavanones, flavones, polymethoxylated flavones and isoflavones. We examined the effects of ten types of flavonoids on the induction of PD-L1 expression in B16F1 cells. The chemical structures of the ten types of flavonoids are shown in Fig. 4. Significant induction of PD-L1 expression in B16F1 cells by nobiletin, genistein and daidzein was observed (Table 1). Among these flavonoids, genistein is a strong inducer of PD-L1 expression in B16F1 cells.

DISCUSSION

In this study, we found that the soy isoflavone genistein enhances IFN-γ-induced PD-L1 expression on B16F1 melanoma cells by flow cytometric analysis, Western blot analysis and quantitative PCR analysis (Figs. 1 and 2). To our knowledge, this study is the first study showing that flavonoids can positively regulate PD-L1 expression in a tumor.

Much attention has been paid to components that can regulate the expression of PD-L1 molecules because PD-1/PD-L1 interaction is crucial for tumor immunity. Some food-derived components have been shown to decrease the expression of PD-L1 on tumor cells. It was shown that apigenin and luteolin suppress IFN-γ-induced PD-L1 expression in human tumor cells *in vitro*.[14,15] Apigenin, luteolin and curcumin also suppressed the growth of tumors *in vivo* mouse models. The results of those studies suggested that inhibition of tumor growth *in vivo* is mediated by the PD-1/PD-L1 pathway.

We found that the flavonoids genistein positively regulates PD-L1 expression (Figs. 1 and 2). PD-L1 expression has been shown to be regulated by many mechanisms. It is known that a signal for cell stress or cell damage induces PD-L1 expression.[16] In addition to the cell damage signal, IFNs including type I (IFN-α/β) and type II (IFN-γ) also induce PD-L1 expression.[17,18] For the mechanism by which IFNs induce PD-L1 expression, it has been shown that IRF-1 binds to the promoter region of PD-L1, resulting in activation of the JAK/STAT pathway. In melanomas, renal cell carcinomas and squamous cell carcinomas, STAT3, but not STAT1, plays a in for the induction of PD-L1 expression by IFNs.[13] We investigated STAT1 and STAT3 mRNA expression and found mRNA expression of both STATs is significantly increased by treatment with genistein (Fig. 3). We examined the effect of gen-

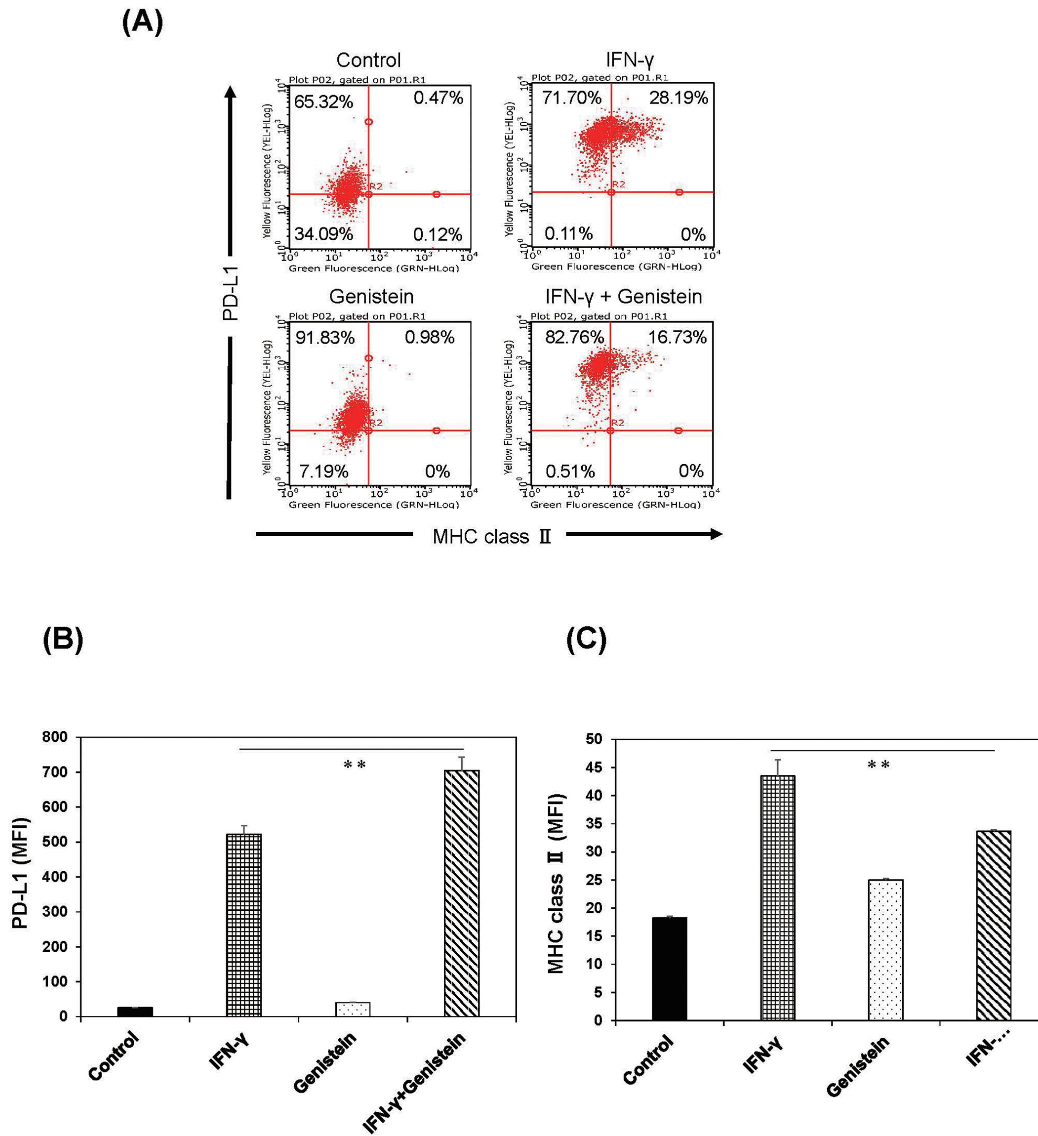

Fig. 1. Genistein Enhances PD-L1 but not MHC Class II Expression in IFN-γ-Treated B16F1 Melanoma Cells

B16F1 cells were treated with 25 U/ml of IFN-γ and genistein for 24 h. Expression of PD-L1 and MHC class II molecules was determined by flow cytometric analysis. Representative expression profiles are shown in Fig. 1 A. Fig. 1 B and C show the MFIs for PD-L1 and I-A molecules, respectively. Data are shown as means ± SD. ** $p < 0.01$.

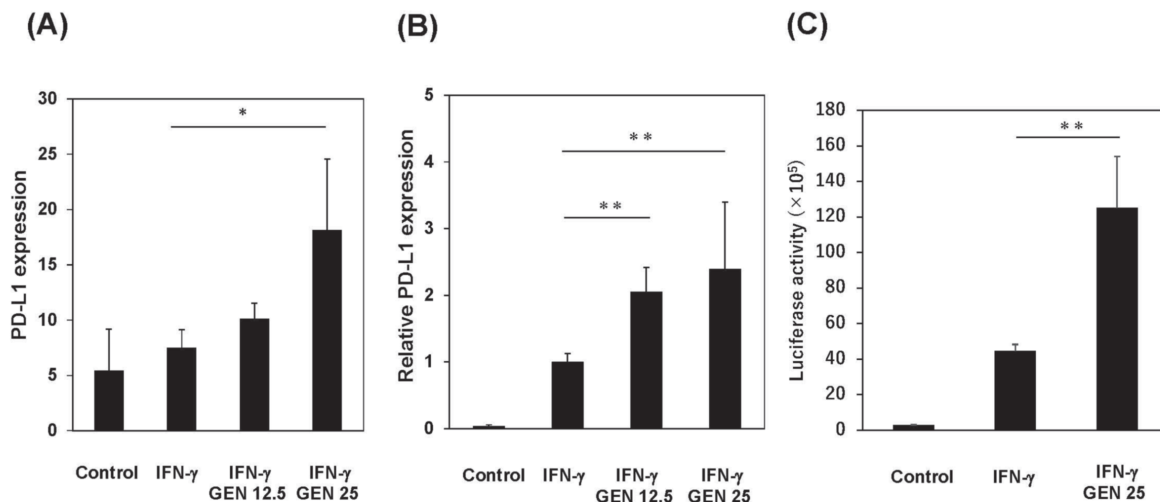

Fig. 2. Genistein Enhances PD-L1 Protein Expression and mRNA Expression in IFN-γ-Treated B16F1 Melanoma Cells

B16F1 cells were treated with 25 U/ml IFN-γ and genistein (12.5 or 25 μM) for 24 h. PD-L1 expression was determined by a Western blot assay (A) and quantitative PCR assay (B). PD-L1 promoter activities after treatment with 25 IU/ml of IFN-γ and/or 25 μM of genistein were determined by a reporter assay as described in the materials and methods section (C). Data are shown as means ± SD. * $p < 0.05$, ** $p < 0.01$.

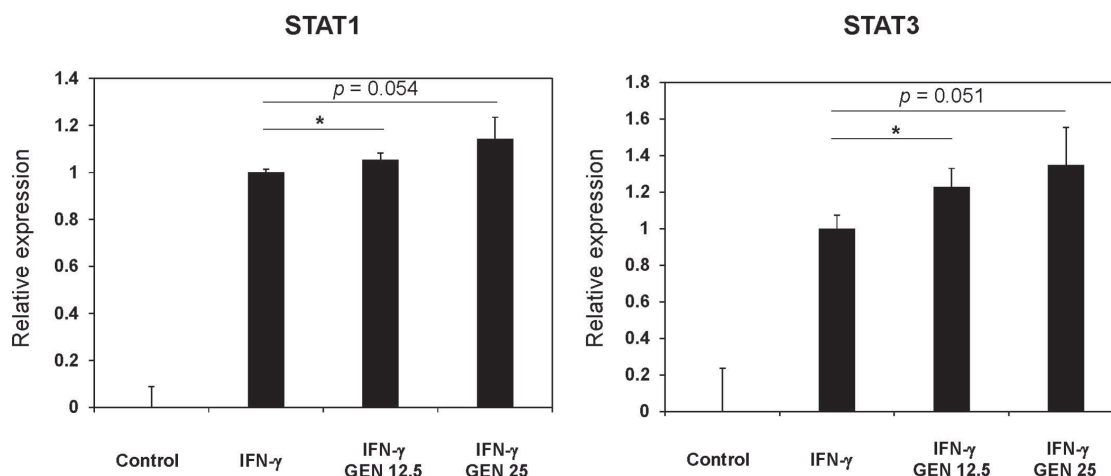

Fig. 3. STAT1 and STAT3 mRNA Expression in B16F1 Melanoma Cells Treated with Genistein

B16F1 cells were treated with 25 U/ml of IFN-γ and 25 µM of genistein for 24 h. STAT1 and STAT3 mRNA expression in B16F1 melanoma cells were determined by a quantitative PCR assay. Data are shown as means ± SD. * $p < 0.05$.

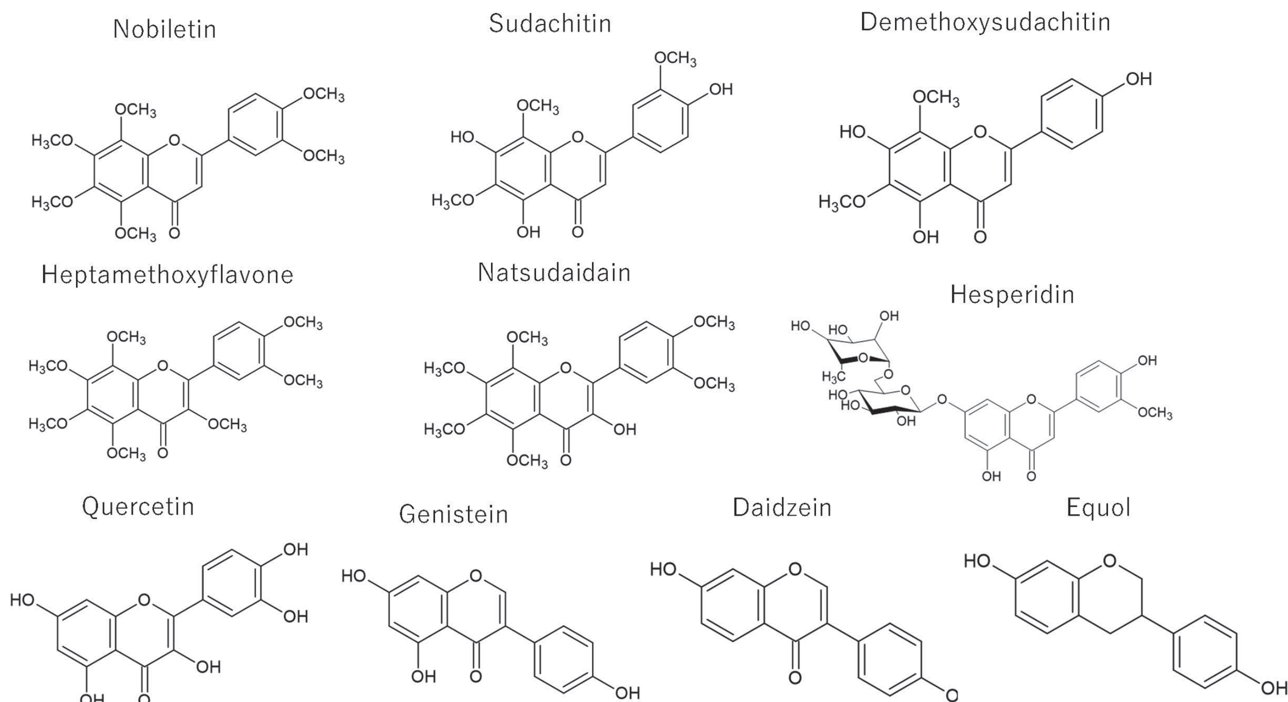

Fig. 4. Chemical Structures of the Ten Types of Flavonoids Used in This Study

istein on expression of PD-L1 by a reporter assay (Fig. 2 C). Since it was found that treatment with genistein enhanced luciferase activity, genistein regulates the expression of a transcription factor that can bind to – 2kb of the PD-L1 gene promoter region. However, it is not known what type of transcription factor that regulates PD-L1 is indued by genistein.

We compared the effects of various types of flavonoids on PD-L1 expression in IFN-γ-treated B16F1 cells. Some of the flavonoids examined in this study enhanced the expression of PD-L1 in B16F1 cells (Table 1). The exact mechanism was not revealed in this study. Nobiletin, sudachitin, demethoxysudachitin and heptamethoxyflavone are polyethoxylated flavonoids that each contain more than two methoxy groups. The numbers of methoxy groups in demethoxysudachitin, sudachitin, nobiletin and heptamethoxyflavone are two, three, four and six, respectively. Since the extends of induction for PD-L1 expression by these polymethoxylated flavonoids were similar (Table 1), the number of methoxy groups might not directly affect the expression of PD-L1.

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Table 1. Expression of PD-L1 in B16F1 Cells Treated with Flavonoids

Mean fluorescence intensity of PD-L1	
Not treated	29.5 ± 0.5[1]
IFN-γ	712.4 ± 31.0
IFN-γ + Genistein[2]	965.4 ± 31.4*
IFN-γ + Nobiletin	842.5 ± 11.5*
IFN-γ + Sudachitin	849.0 ± 57.7
IFN-γ + Demethoxysudachitin	774.0 ± 53.8
IFN-γ + Heptamethoxyflavone	850.7 ± 48.1
IFN-γ + Natusudaidain	716.6 ± 8.3
IFN-γ + Hesperidine	581.9 ± 19.9
IFN-γ + Quercetin	771.0 ± 16.9
IFN-γ + Daidzein	871.2 ± 8.3*
IFN-γ + Equol	622.6 ± 1.4

[1]Mean ± SD
[2]Concentration of each flavonoid is 25 μM
*$P < 0.05$

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Conflict of interest The authors declare no conflict of interest.

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